

Reasons of Unknown HPLC Peaks during Hemoglobin Analysis for Patients with Beta Thalassemias

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Introduction

Beta-thalassemias (β -thal) are inherited quantitative disorders of haemoglobin (Hb) leading to underproduction of the beta globin chains of hemoglobin. In β -thal, the synthesis of normal α globin chains from the unaffected α globin gene continues as normal, resulting in the accumulation within the erythroid precursors of excess free α globin chains, which precipitate in the red cell precursors in the bone marrow forming inclusion bodies. Excess free alpha globin chain precipitate as insoluble hemichrome on the inner surface of red cell membrane (membrane-bound), forming inclusions called micro-Heinz bodies or occur as free cytosolic (soluble) hemichrome [1]. Among other names (free alpha hemoglobin, hemoglobin subunit alpha, alpha-globin, alpha-1 globin, alpha-2 globin, unbound alpha globin chain, free alpha globin chain, free alpha haemoglobin chain, hemoglobin alpha-1 chain and hemoglobin alpha 1 globin), free alpha haemoglobin (free α -Hb) is the preferred name [2,3]. A small but reproducibly detectable, excess of α -globin mRNA is present in normal erythroblasts in β -thal. However, β -globin mRNA is translated more efficiently than α -globin mRNA. These counterbalancing forces result in approximately equal synthesis of α - and β -globin polypeptide chains. In normal reticulocytes, α - and β -globin chains production is synchronous, though there is a very small excess of alpha chain synthesis [3]. The pathophysiology of β -thal is the consequences of the accumulation of excess, free α -Hb in erythroid precursors in bone marrow and peripheral red blood cells forming inclusion bodies abundant enough to be visible by light microscope in red cells precursors and mature forms. The severity of β -thal is mainly correlates with the extent of imbalance between α - and non- α -globin chains and the amount of the free α -Hb pool in the erythrocytes rather than the underproduction of Hb. The erythroid cells capacity to detoxify and remove the damaging α -Hb inclusions protein via quality control pathway are exceeded in red cells of individual with severe forms of β -thal [4]. The major problem in β -thal is accumulation of excess free globin chains (α -Hb) in the erythrocytes precursors in the bone marrow and peripheral erythrocytes forming inclusion bodies, thus, patients with β^0 thalassemia is clinically worse than patients with β^+ thalassemia, and there is a general trend in this direction [5,6]. In β -thal trait there is a two-fold increase in the synthesis of alpha globin and the ratio of alpha to non-alpha globin in individual with β -thal intermedia is three to four folds increased compared to healthy individual with marked chain imbalance in β -thal major [3]. Cation-exchange High performance liquid chromatography (CE-HPLC) is used for hemoglobin variants determination. In this system, the Hb variants are pre-defined with; Unique elution/ retention times (the

time elapses from the sample injection to the apex of the elution peak), peak or curve shapes and peak height / amplitudes (Area). The currently available HPLC software for Hb variants analysis (Variant II*, Bio-Rad Laboratories, β Thalassemia Short program), does not integrate any portion that elute before 0.75 min. Peak that elutes at a non-predefined / non-Integrated retention time is labeled as unknown peak. The unknown peaks are identified only by visual analysis of the chromatography (c-gram). Moreover, the calculated total percentages of the Hb measured by Variant II* HPLC are not always 100 percent [7,8]. Separation of hemoglobin variants by HPLC analyzer is due to the affinity of the hemoglobin cation to the poly aspartic acid-coated particles of silica contained in a separation column. For separation, the diluted blood is passed under pressure through the column and then the eluted fraction passed to detector where the amount of the eluted fraction is measured and expressed as a peak. Thereafter, the eluted fraction of the solution is drained under no pressure into the waste bottle [9,10]. We have noted the occurrence of unknown early-eluting peaks within the first-minute retention time during high-performance liquid chromatography (HPLC) analysis of hemoglobin (Hb) in patients with different clinical phenotypes of β -thal. We noticed the low-amplitudes or absence of the early-eluting unknown peaks during HPLC analysis of blood from healthy individual and even in the presence of other structural Hb variants such as Hb-S. Moreover, the peak's amplitudes are comparable with the levels of Hb-F or Hb-A2 and that the retention time of these unknown HPLC peaks is close to where Hb-H & Hb-Barts eluted during HPLC analysis (Figure 1). Hb Barts (γ_4) and Hb-H elutes from the separation column prior to the start of integration, and appear as fast-eluting peaks in HPLC. Because the α -Hb is composed of monomers and dimers of free alpha globin chain, it could elutes and appear as peaks at early retention time on HPLC analysis similar to the pattern of Hb-H and Hb-Bart as each of them is composed of one type of globin chain, alpha, beta & gamma consequently [11-14]. The currently proposed reasons of the unknown peaks are: Hb-Bart, Hb-H, altered / acetylated Hb-F in neonate (Hb-F1), bilirubin and Injection artifacts [7-15]. Recombinant alpha-hemoglobin stabilizing protein was successfully used to measure the free alpha-hemoglobin (α -Hb) in hemolysate of patients with β -thal and healthy individuals [16]. There is lack of biomarker that conveys diagnostic and / or prognostic values in the management of β -thal of various clinical phenotypes. Understanding the significances of free α -Hb was recommended [3,16].

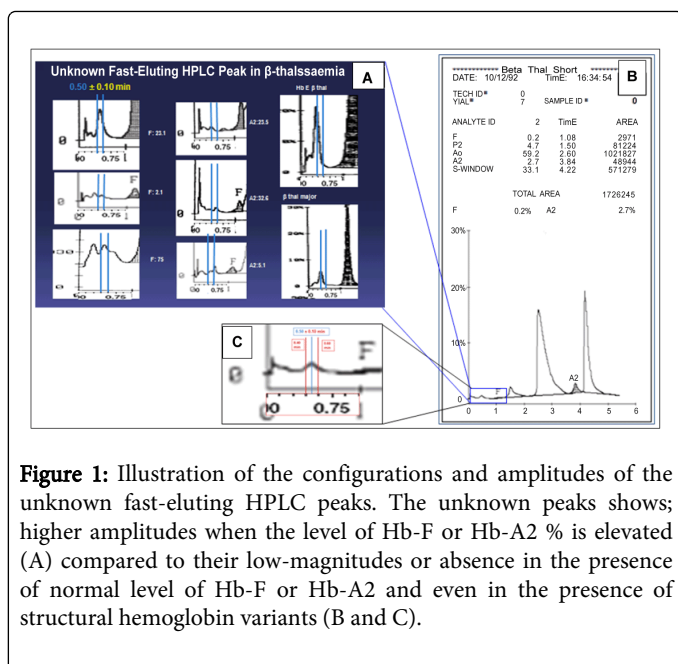


Figure 1: Illustration of the configurations and amplitudes of the unknown fast-eluting HPLC peaks. The unknown peaks shows; higher amplitudes when the level of Hb-F or Hb-A2 % is elevated (A) compared to their low-magnitudes or absence in the presence of normal level of Hb-F or Hb-A2 and even in the presence of structural hemoglobin variants (B and C).

Conclusion

Configuration analysis of the unknown peaks eluted during the HPLC analysis of hemoglobin for the manifestation of free α -Hb in patients with β -thal of various clinical phenotypes including Hb-E β -thalassaemia syndrome needs to be formulated.

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